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Determination of Serum Calcitonin by Immunometric Two-Site Assays in Normal Subjects and Patients with Medullary Thyroid Carcinoma

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Summary: We evaluated the clinical usefulness of a commercial two-site immunoassay for calcitonin, which is based on two monoclonal antibodies against distinct epitopes of calcitonin. The potential lower detection limit of the assay was 2 ng/l. Inter-assay and intra-assay variation were both < 11%. No cross reaction with calcitonins from different species (salmon and eel calcitonin) was found. Of the various fragments of human calcitonin, only the 11–32 fragment could be detected.

Basal calcitonin concentrations in normals (n = 69) ranged from < 2 ng/l to 50 ng/l. Basal calcitonin concentrations in 30% of the men (< 2–48 ng/l) and 51% of the women (< 2–10 ng/l) were undetectable. Stimulation of calcitonin with pentagastrin (0.5 μg/kg body weight) in normal subjects was followed by an increase in calcitonin in all men (n = 17) and in 60% of the women (n = 12). The upper reference value for pentagastrin-stimulated calcitonin concentrations for all 29 normals was 78 ng/l (defined as the 95th percentile): 79 ng/l for men, and 50 ng/l for women. Using a different immunoradiometric two-site assay, a similar percentage of "positive" responses to pentagastrin in normals was obtained (20 out of 29).

Four previously thyroidectomized patients with medullary thyroid carcinoma had normal basal, but pathological stimulated calcitonin values at follow-up. At reoperation, cervical lymph nodes with tumour tissue were removed. Postoperatively, the patients had basal and stimulated calcitonin concentrations within the limits of the established reference range.

In conclusion, reference ranges for basal and pentagastrin-stimulated calcitonin concentrations have been established for an immunometric two-site assay. This allows a safer discrimination between normal and pathologically stimulated calcitonin concentrations. This may lead to a better definition of cured patients and perhaps an earlier diagnosis of medullary thyroid carcinoma in familial screening.

Introduction

For diagnosis, screening and follow-up of medullary carcinoma of the thyroid, calcitonin is commonly determined in unextracted samples of plasma or serum by radioimmunoassay with polyclonal antibodies (1, 2). Measurement of calcitonin by conventional radioimmunoassays, however, still presents problems. Thus, multiple heterogeneous forms of circulating immunoreactive calcitonin, which are variably detected by polyclonal antisera, mainly account for the wide range of reported reference values, and their high background concentrations may mask significant changes in calcitonin concentration (3–5). Some of these problems have been overcome to some extent by the improvement and evaluation of analytical procedures, but physiological concentrations of plasma calcitonin are low and still usually undetectable by conventional radioimmunoassays (2, 6). Therefore small changes in circulating calcitonin remain below the detection limit and may be missed. This is of particular concern in screening for patients with familial medullary thyroid carcinoma, in which circulating

basal calcitonin concentrations often do not differ from the reference range. In this setting provocative tests with pentagastrin or calcium infusion are still necessary (2, 7).

The new generation of two-site immunoassays using two monoclonal antibodies against distinct epitopes of human calcitonin have largely improved both the sensitivity and the specifity of calcitonin determination (8, 9). These assays are now commercially available, but little is known regarding their clinical superiority, particularly the reference range for pentagastrin tests. Therefore we established a reference range for pentagastrin-stimulated calcitonin concentrations and evaluated the clinical usefulness of two, new, commercially available immunometric assays (one immunoradiometric assay and one enzyme immunometric assay).

Materials and Methods

Two-site assays for calcitonin

Calcitonin concentrations were determined by a solid phase two-site enzyme immunometric assay (Medgenix, Düsseldorf, Germany). Calcitonin is captured by a monoclonal antibody directed against it and immobilized on microtitre plates. Detection is performed using a second monoclonal antibody labelled with horseradish peroxidase. The assay requires incubation of the sample overnight at 4 °C, followed by a short incubation of 15 min after five washing steps. Serum, heparinized plasma or EDTA plasma may be used without extraction. The lower potential detection limit of the assay was determined and defined as the point of the calibration curve 3 standard deviations above B_0 (based on 16 measurements of the 0-standard). To obtain intra- and inter-assay variations, samples with known concentrations of calcitonin (10–613 ng/l) were repeatedly measured in eight separate assays and simultaneously measured eight times in one assay. Samples from five patients with medullary thyroid carcinoma and high concentrations of basal calcitonin were serially diluted in calcitonin-free medium (provided with the assay) to estimate linearity of the assay in the range of measurement (2–1000 ng/l). The specificity was tested by assaying various concentrations of calcitonin from salmon and eel and various concentrations of different fragments of human calcitonin.

The fragments of human calcitonin and eel calcitonin were a gift from Dr. *Kabay*, Ciba Geigy, Basel, Switzerland; salmon calcitonin was a gift from Sandoz, Nürnberg, Germany.

Additionally, basal and pentagastrin stimulated calcitonin concentrations in 29 normals were also measured by a commercially available immunoradiometric two-site assay of calcitonin (ELSA-hCT, CIS, Dreieich, Germany). The assay is based on two monoclonal antibodies (8) against calcitonin (calcitonin sequences 11–17 and 24–32), uses a single incubation step and has a detection limit of 2 ng/l.

Samples

Basal calcitonin concentrations were determined in 69 healthy subjects (26 women, 43 men, age 20–70 years) and in 9 thyroidectomized patients. In 29 healthy volunteers (17 men, 12 women, age 20–50 years), who gave informed consent, a pentagastrin stimulation test was performed: 0.5 µg pentagastrin (Gastrodiagnost®, Merck, Darmstadt, Germany) per kg body weight was injected intravenously within 5 s and blood samples were drawn at 5 minutes before and 0, 2, 5, 10, 20 and 30 min after injection.

In four patients with histologically confirmed medullary thyroid carcinoma, both the basal and the pentagastrin-stimulated concentrations of calcitonin were determined before and after reoperation.

Results

Assay characteristics

The lowest detectable calcitonin concentration was 1.7 ng/l (for practical reasons 2 ng/l). This was comparable with the detection limit of 2 ng/l of the immunoradiometric assay used for comparison (CIS). The inter-assay variation was 5.8% for 25 ng/l, 9.9% for 134 ng/l and 7.2% for 613 ng/l. The intra-assay variation was 11.2% for 10 ng/l, 5.8% for 25 ng/l and 2.4% for 134 ng/l. The recovery of the assay proved to be linear over a braod range of concentrations (2–1000 ng/l) as tested by dilution of samples from patients with known medullary thyroid carcinoma (fig. 1). In order to test the specificity, the cross-reactivities of different fragments of human calcitonin and calcitonin of different species were tested. The intact molecule 1–32 and the fragment 17–32 cross-reacted (99.6% and 120%, respectively). The human calcitonin-fragments 1–10 and 11–23 were not detectable up to concentrations of 300 pmol/l. Salmon calcitonin and eel calcitonin displaced the human 1–32 tracer by up to 8% at concentrations of 300 pmol/l, i.e. the assay is 200-fold less sensitive for fish calcitonin than for human calcitonin.

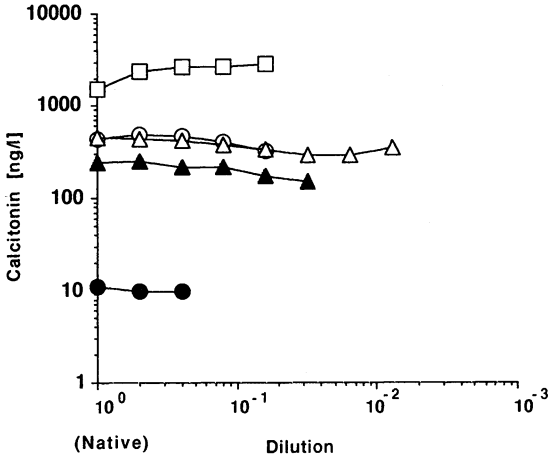

Fig. 1. Serial dilution of samples from patients with medullary thyroid carcinoma showing linearity in the range of 10–2000 ng/l; y-axis shows calcitonin concentration, x-axis shows the dilution factor.

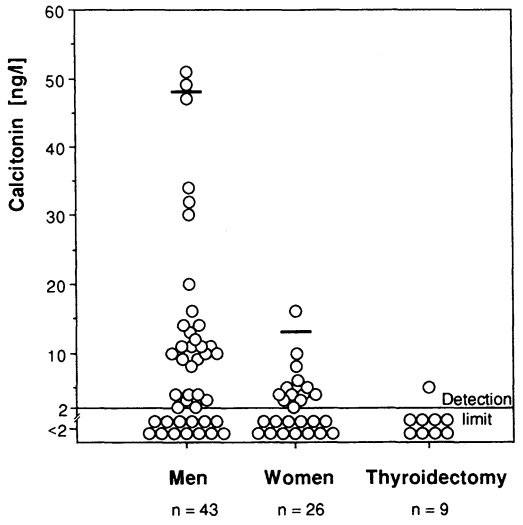

Fig. 2. Basal concentrations of calcitonin in 69 healthy subjects (43 men, 26 women) and 9 thyroidectomized patients. The horizontal line indicates the 95th percentile for both women and men.

Basal calcitonin concentrations

Basal calcitonin concentrations were detectable (> 2 ng/l) in 60% of 69 healthy subjects, and ranged from < 2 ng/l to 50 ng/l. The mean value was 8.4 ± 13 ng/l (undetectable value set as 2 ng/l), but the values were not normally distributed. The median was 4 ng/l with a 95th percentile of 48 ng/l as the upper limit. Values were higher in men than in women, the 95th percentile for women being 10 ng/l and for men 49 ng/l. In the male group 23% of all values were undetectable, whereas in the female group 42% of the values were below the detection limit (fig. 2). Serum values from eight of nine thyroidectomized patients were below the detection limit.

Calcitonin concentrations after stimulation

In 29 healthy persons a rapid intravenous injection of pentagastrin (0.5 µg/kg body weight) resulted in an acute rise of calcitonin concentrations within 2–5 min, which declined to basal values 10 minutes after the injection (fig. 3). The calcitonin concentrations after pentagastrin stimulation ranged from undetectable (< 2 ng/l) to 135 ng/l. In all men, the stimulated calcitonin concentrations were within the detectable range, whereas basal calcitonin concentrations were only detectable in 77%. The percentage of women with detectable calcitonin concentrations increased from 49% (basal) to 60% (after pentagastrin stimulation). Since the values were not equally distributed, the 95th percentile was used to establish the upper limit of normal. The 95th percentile for stimulated calcitonin concentrations was 50 ng/l for women, 79 ng/l for men and 78 ng/l for both (tab. 1).

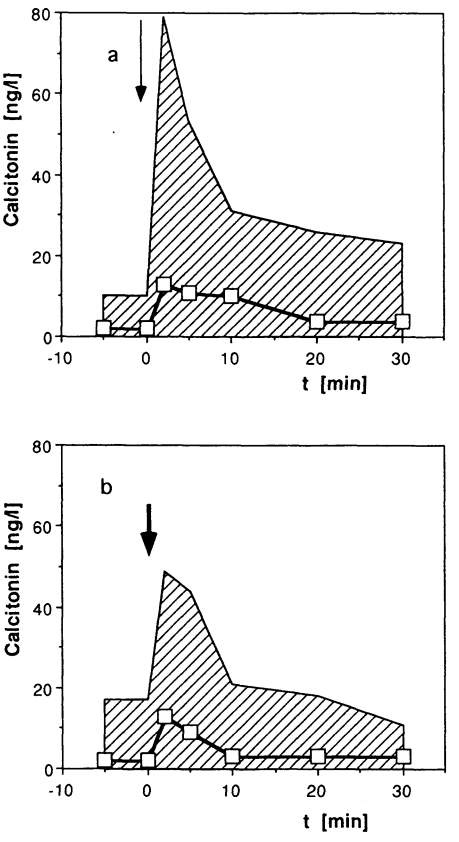

Fig. 3. Pentagastrin stimulation (↓) in normals (n = 29); a) men (n = 17), b) women (n = 12). The 95th percentile (shaded area) and the median (open squares) are given.

Tab. 1. Comparison between immunoenzymetric (Medgenix) and immunoradiometric (CIS) assays for calcitonin in 29 normals. Basal calcitonin concentrations and values after pentagastrin-stimulation are shown in ng/l.

Calcitonin assays		Men (n = 17)		Women (n = 12)	
		Basal	After stimulation	Basal	After stimulation
Immuno-enzymetric assay	95th percentile	10.7	79	17	49.5
(Medgenix®)	Median	2.1	13.1	2.3	13.4
Immuno-radiometric assay	95th percentile	6.2	66.3	15.3	24.4
(CIS®)	Median	2.3	6.8	2.7	5.4

Using the immunoradiometric two-site assay for the same 29 pentagastrin stimulation tests, a similar increase due to pentagastrin stimulation was found (tab. 1). "Positive" responses to pentagastrin (defined as an increase from < 2 ng/l to > 2 ng/l or more than a two-fold increase in detectable values) were detected with the same frequency by both assays (19/29 in the

enzyme immunometric assay vs. 20/29 in the immunoradiometric assay). The correlation coefficient between the basal and stimulated calcitonin concentrations of both assays was r = 0.7.

Calcitonin concentrations in patients with medullary thyroid carcinoma

In four patients with medullary thyroid carcinoma who underwent total thyroidectomy, basal calcitonin concentrations, as determined with the enzyme immunometric assay, were within the normal range, but a pathological increase after pentagastrin stimulation was observed (range: 443 – > 900 ng/l), which clearly exceeded the reference range (tab. 2). After reoperation, calcitonin concentrations before and after pentagastrin stimulation were within the reference range. Apparently these patients were cured with respect to biochemical criteria; only one patient still showed slightly increased calcitonin concentrations after pentagastrin stimulation.

Tab. 2. Basal calcitonin concentrations and values after pentagastrin stimulation of thyroidectomized patients with medullary thyroid carcinoma before and after reoperation.

Patient Sex	Preoperative calcitonin concentrations (ng/l)		Postoperative calcitonin concentrations (ng/l)	
	Basal	Pentagastrin stimulation	Basal	Pentagastrin stimulation
1 ♂	74	918	3	2
2 ♂	38	922	1	14
3 ♀	7	443	2	131
4 ♂	23	935	12	73

Discussion

The tested enzyme immunometric calcitonin assay (Medgenix) provides rapid and reliable results without time consuming extraction methods. The lower detection limit of 2 ng/l is better than the lower detection limit of the standard radioimmunoassays and equivalent to that of the first immunometric assay for calcitonin using monoclonal antibodies described by *Motte* et al. (8, 9). The assay antibodies did not cross-react significantly with calcitonin from other species or human calcitonin fragments, except for human calcitonin fragment 11 – 32. Apparently both antibodies recognize epitopes within the sequence 11 – 32 of the calcitonin molecule or fragments which are longer than human calcitonin 10 – 32. This is in agreement with the theoretical expectation based on the utilization of monoclonal antibodies against the epitopes of amino acids 11 – 13 and 30 – 32. Since the latter epitope is only accessible on the mature form of the hormone (8), the assay should be specific for the mature circulating form of calcitonin.

Despite the high sensitivity of the assay, only 65% of the basal calcitonin concentrations in control subjects were detectable. Consistent with the fact that, in general, women have lower calcitonin concentrations (10) and with the results of *Perdrisot* et al. (11), the percentage of undetectable values is higher in women. With the modified assay of *Motte* et al., 83% of control persons had undetectable basal concentrations of calcitonin (< 5 ng/l) (11, 12). Comparing the percentage of undetectable calcitonin concentrations is difficult, however, as no information is given about the sex and age of the control group.

In our study, all men responded with a measurable increase of calcitonin concentrations after pentagastrin stimulation. In women, the percentage of detectable values increased from 49% to 60%, so that 40% were still undetectable. In agreement with our results, *Perdrisot* et al. (11) also found a measurable increase of calcitonin concentrations in 50% of his subjects. The immunoradiometric assay (CIS) revealed increases during pentagastrin tests in normal subjects (20/29), which were similar to those measured with the enzyme immunometric assay (19/29), according to our definition of "positive" pentagastrin tests. Using the same assay, *Weissel* et al. (13) even reported measurable basal and stimulated calcitonin concentrations in all subjects tested, but his number of control subjects was very small (n = 4). Interestingly the absolute increases in calcitonin concentrations in our volunteers were much higher than those reported by *Perdrisot* et al. or *Weissel* et al. (11, 13). We found a maximum of 135 ng/l in one healthy man after stimulation, whereas the maximal increase of calcitonin reported by *Perdrisot* et al. was only 30 ng/l. There might be several explanations for this discrepancy. The enzymatic assay might be prone to unspecific interferences or due to slightly modified assay protocols (13). However, it is difficult to compare absolute concentrations of calcitonin after stimulation since a slow injection rate of pentagastrin (over 3 min) (11) has been used in the literature. We found the most distinct increase of calcitonin 2 min after a bolus injection of pentagastrin within 5 s; therefore the discrepancy is at least to some extent due to a slower injection rate of pentagastrin.

A major advantage of the tested assays is the precise definition of the upper normal limit for stimulated

calcitonin concentrations. We used the 95th percentile to define a reference range for stimulated calcitonin concentrations in normal subjects, as the values after stimulation were not equally distributed. In 4 out of 20 normal subjects deviating peak concentrations have been reported in the literature after stimulation with calcium combined with pentagastrin (14). We did not observe any widely deviating peak concentration in our study.

Four of our previously operated patients with medullary thyroid carcinoma had basal calcitonin concentrations within the normal range; the increase after pentagastrin, however, was rapid and largely exceeded the new defined reference range. After localization of tumour tissue in the neck and reoperation the patients showed a decrease in basal calcitonin concentrations and after stimulation and were cured with respect to the calcitonin concentration in serum (15). The use of more sensitive calcitonin assays with a well defined reference range for basal calcitonin concentrations and after stimulation has shown that the cure rate of medullary thyroid carcinoma by total thyroidectomy is not as high as previously thought based on results from radioimmunoassays. Similar results have been reported by *Kaplan* et al. (16) from the screening of multiple endocrine neoplasia 2A families. In archived sera from patients who had undergone pentagastrin tests, the more sensitive assays showed abnormally elevated calcitonin concentrations in three patients with C-cell hyperplasia 3–4 years or more before any abnormality was detected by the standard calcitonin radioimmunoassay. By using an immunoradiometric assay, *Guilloteau* et al. (12) found, in eight affected family members with medullary thyroid carcinoma, a higher percentage of pathological basal (5/8) or stimulated (8/8) calcitonin concentrations than with a conventional radioimmunoassay (1/8 and 4/8 respectively). These assays need to be tested more extensively in familial screening of medullary thyroid carcinoma, but the present results favour the use of highly sensitive two-site assays for calcitonin for detecting any C-cell disease during familial screening and during postsurgical follow-up. The established reference ranges enable the detection of pathological calcitonin concentrations in the pentagastrin test.

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